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A minimally invasive tool to study immune response and skin barrier in children with atopic dermatitis

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Stratum corneum analysis provide a minimal invasive tool to study immune response and skin barrier in atopic dermatitis children

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Short title SC tape stripping in AD children with light and dark skin types

What's already known about this topic?

AD affects children of all skin types, but most research is focused on Caucasians with light skin types. Studies investigating biomarkers in AD patients with dark skin types are lacking. Tape stripping can be used for analysis of skin barrier and immune response biomarkers in AD skin.

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What does this study add?

SC tape stripping is suitable to study a broad panel of biomarkers in AD children. We show differences in skin barrier and immune response biomarkers between lesional, non-lesional and control skin. Immune response biomarkers are similar in light and dark skin types. However, NMF levels differ between skin type II and VI, hinting towards a possible different pathogenesis in AD between light and dark skin types.

Summary

Background Atopic dermatitis (AD) affects children of all skin types, most research is focused on Caucasians with light skin types. Studies investigating biomarkers in AD patients with dark skin types are lacking.

Objectives To explore skin barrier and immune response biomarkers in stratum corneum (SC) tape strips from AD children with different skin types.

Methods Tape strips were collected from lesional and non-lesional forearm skin of 53 AD children and 50 control children. We analysed 28 immunomodulatory mediators, as well as natural moisturizing factors (NMF) and corneocyte morphology with atomic force microscopy.

Results IL1 β , IL18, CXCL8, CCL22, CCL17, CXCL10 and CCL2 were significantly ($p < 0.05$) higher in lesional AD skin versus non-lesional AD skin, while IL-1 α showed the opposite trend. CXCL8, CCL2 and CCL17 showed association with oSCORAD. NMF levels showed a gradual decrease from healthy skin to non-lesional and lesional AD skin. This gradual decreasing pattern was observed in skin type II but not in skin type VI. Skin type VI showed higher NMF levels in both non-lesional and lesional AD skin compared to skin type II. Corneocyte morphology was significantly different in lesional AD skin versus non-lesional AD and healthy skin.

Conclusions Minimally invasive tape stripping is suitable for determination of many inflammatory mediators and skin barrier biomarkers in AD children. This explorative study shows differences between AD children with skin type II and skin type VI in NMF levels, suggesting that some aspects of pathophysiological mechanisms may differ in AD children with light versus dark skin types.

Keywords atopic dermatitis, stratum corneum analysis, biomarkers, children, natural moisturizing factor, skin barrier, skin type differences

Abbreviations

AD	Atopic dermatitis
AFM	Atomic force microscopy
AMC	Academic Medical Centre, Amsterdam, the Netherlands
BH	Benjamini-Hochberg
CCL	CC chemokine ligand (-2,-3,-4,-11,-13,-17,-18,-22,-26)
CTRL	Healthy skin of controls
CXCL	CXC chemokine ligand (-8,-10)
DTI	Dermal Texture Index
FDR	False discovery rate
FLG	Filaggrin
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HIS	Histidine
IL	Interleukin (-1,-1 α ,-1 β ,-2,-4,-5,-6,-7,-10,-12p40,-12p70,-13,-15,-16,-17A,-18)
INF	Interferon (- γ)
IQR	Interquartile Range (Q1-Q3)
L	Lesional AD skin
MWU	Mann Whitney U test
NL	Non-lesional AD skin
NMF	Natural Moisturizing factor
oSCORAD	Objective SCORAD score
PCA	Pyrolidone-5carboxylic acid
SCORAD Score	Scoring atopic dermatitis score, clinical tool for scoring AD severity
SC	Stratum corneum
SD	Standard deviation
SEM	Scanning electron microscopy
TEWL	Trans-epidermal water loss
Th2	T helper cell type 2
TNF	Tumor necrosis factor (- α)
UCA	Urocanic acid
VP	Villus-like projections
W-test	Wilcoxon test

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease affecting 10-20% of children (1). AD affects children of all skin types and seems more prevalent in Asian and children with dark skin types (2, 3). However, most research is focused on Caucasians with light skin types and studies investigating biomarkers in AD patients with dark skin types is lacking. Data on physiological skin properties in different skin types is conflicting. Darker skin types seem more resistant to irritants than lighter skin types (4), but trans-epidermal water loss (TEWL) seems greater in dark skin types compared to lighter skin types (5). Furthermore, the stratum corneum (SC) of dark skin types shows higher lipid content and seems more compact due to greater intercellular cohesion. (6, 7). Nevertheless, the SC thickness is demonstrated to be similar for all different skin types (8). The SC and filaggrin (FLG) have a crucial role in forming the epidermal barrier (9). FLG degradation products, such as histidine

(HIS), pyrrolidone-5carboxylic acid (PCA) and urocanic acid (UCA), are the main source of natural moisturizing factors (NMF) and are responsible for mechanical properties, skin hydration and inflammatory response in skin (10).

In AD patients, abnormalities in corneocyte morphology such as prominent villus-like projections (VP) have been observed using high-resolution atomic force microscopy (AFM) (11, 12). Although the nature of these VP is not well understood, VP can be used to measure skin barrier function (11). The number of VP per investigated corneocyte area is expressed as the Dermal Texture Index score (DTI). In AD children, a strong negative association was found between NMF levels and corneocyte morphology abnormalities measured by DTI (13). Moreover, these DTI values in AD children with FLG mutations might explain phenotypic differences in AD (13).

Serum inflammatory profiles are investigated to gain more insight in immune pathways and future personalised therapeutic options. Cytokines (IL4, CXCL8, IL13) and chemokines (CCL2, CCL17, CCL18, CCL22) are correlated with AD severity in adults (14-16). However, obtaining blood samples and skin biopsies is difficult in children, therefore detailed data of skin samples are lacking. A minimally invasive method to detect biomarkers would provide an essential tool in dissection of childhood AD into disease endotypes, and predicting or monitoring flares and therapy efficacy in childhood AD.

Our aim was to use the minimally invasive method of SC tape stripping to study a large array of SC biomarkers including immunomodulatory mediators, NMF and DTI in AD children compared to children with healthy skin. We assessed differences in these biomarkers in light skin types compared to dark skin types, and in children with and without FLG mutations.

Patients and methods

AD patients and healthy controls

Children (0-12 years) diagnosed with AD according to the UK working party criteria (17-19) were recruited (2015-2016) from the paediatric and dermatology outpatient clinic in a tertiary referral centre, Academic Medical Center (AMC). Children treated with systemic corticosteroids, antibiotics, antimycotics or extensive UV exposure 4 weeks prior to tape stripping were excluded, and children were instructed not to use antihistamines (72 hours) and emollients, soap and perfumes on the selected forearm (48 hours). For AD children the forearm skin should not have been treated with topical corticosteroids (1 week). AD severity was assessed using objective scoring atopic dermatitis score (oSCORAD) (20, 21).

Control children (no medical history of inflammatory skin disorders or AD, and no first degree relatives with AD) with healthy skin were recruited from the AMC surgery outpatient clinic. Skin types were categorised according to Fitzpatrick skin phototypes, ranging from I to VI (22). Analyses were limited to skin types II and VI due to low sample sizes of remaining skin types.

SC tape-stripping

The SC was collected, as previously described (23) by tape stripping a visibly non-lesional skin site of the forearm, and in some AD children also from a lesional site of the forearm. Circular adhesive tapes (D-squame® discs, 22 mm diameter, 3.8 cm², Monaderm, Monaco, France) were placed to volar forearm and pressed for ten seconds with a pressure of 225 g/cm² of D-Squame Pressure Instrument D500 (CuDerm Corporation, Dallas, Texas). Sequentially, 6 consecutive tapes from the same skin site were collected. The 3rd and 4th tape strip for determining corneocyte morphology and NMF were stored at -20°C, all other tape strips were stored at -80°C until analysis.

FLG genotyping

We analysed the four most prevalent FLG mutations (R501X, 2282del4, R2477X, S3247X) from DNA extracted from a buccal swab as previous described (24, 25).

Immunomodulatory mediators

Levels of 28 immunomodulatory mediators (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, IL-18, IFN-γ, TNF-α, GM-CSF, CXCL8, CXCL10, CCL2, CCL3, CCL4, CCL11, CCL13, CCL17, CCL22 and CCL26) were determined in the 5th and 6th tape strip, representing the SC middle part, using the MESO QuickPlex SQ 120 assay (MSD, Rockville, Md., USA) on samples extracted by ultrasonification (15 min) with 0.5 ml of phosphate-buffered saline containing 0.05% Tween 20 and stored at -80 °C according to manufactory protocol. Samples under the limit of detection were substituted for by a value of half the level of the detection limit. Levels were corrected by total protein content, as determined using Pierce Micro BCA protein assay kit (Thermo Fischer Scientific, Rockford, IL, USA,).

NMF determination

NMF components PCA, His and UCA (cis- and trans isomer) were extracted with 500 μL of 25% (w/w) ammonia solution, reconstituted in 500 μL water after evaporating ammonia and analysed by HPLC-UV as described previously (23,26). NMF concentrations were also corrected for total protein content and expressed as mmol/g protein.

Corneocyte morphology (DTI)

In a randomly chosen subgroup (16 controls and 21 AD children) with skin type II or VI corneocytes morphology was analysed with AFM as described previously (27, 28). Besides analysis of non-lesional skin, in 13 AD children DTI was also determined in lesional skin. AFM was used on the 3rd tape strip with a Nanoscope III controller and software version 5.30sr3 (Digital Instruments, Santa Barbara, CA, USA) (27). For nano-texture analysis, subcellular scan areas of $(20\text{ }\mu\text{m})^2$ were recorded (13). A recently developed software method can quantitatively determine the VP surface nano-texture on corneocytes. Topographical data of cell surfaces of the tape strips was analysed using Anostic™-method applying custom built, proprietary algorithms (13) enabling assessment of the number of nano-size protrusion. The number of these protrusions per $(20\text{ }\mu\text{m})^2$ unit area has been expressed as the DTI score (13).

Statistical analysis

Data analysis was done by SPSS version 24 (IBM SPSS Statistics for Windows, Armonk, New York, USA) and GraphPad prism® version 7.0 (GraphPad Software, La Jolla, California, USA). Baseline characteristics were analyzed by Student's t-test (for normally distributed continuous data), Mann-Whitney U-test (for nonparametric continuous data), chi-square test (for dichotomous data). Distribution of data was tested by Shapiro-Wilk normality test. Mann Whitney U test (MWU-test) was used for unpaired comparison between groups (healthy versus lesional skin) and Wilcoxon Signed Rank test (W-test) for comparison within the AD group (non lesional versus lesional skin). A two-tailed nonparametric Spearman correlation analysis was used to determine the overall associations between biomarkers and oSCORAD. Multiple linear regression analysis was performed with skin severity as dependent and biomarkers, skin type and FLG mutations as independent variables using stepwise backward selection method. Because of skewed distribution cytokine levels were log-transformed before linear regression analysis. Correction for multiple testing, using the Benjamini-Hochberg (BH) method was conducted for all the resulting p-values, the corrected p-values <0.05 were considered significant.

Results

Patient characteristics

Demographic characteristics are shown in Table 1. Control children were slightly older. AD severity was mild to moderate with a mean oSCORAD of 17.6 (\pm SD 9.7). Within the AD group, FLG mutations were less prevalent in darker skin types, as only 1 child with skin type VI (1/14; 7%) had a FLG

mutation, while 64% (9/14) of skin type II AD children had a FLG mutation. Most children were investigated in summer season (76% controls versus 49% AD children).

Distinct inflammatory immune responses can be detected in SC of AD children

From 28 measured immunomodulatory mediators 13 could quantitatively be determined in SC samples. In 9 of the 13 immunomodulatory mediators significant differences were found between healthy, non-lesional and/or lesional skin. IL1 α was significantly lower ($p<0.05$) in non-lesional AD skin compared to healthy skin of controls, which was even more pronounced ($p<0.01$) when compared to lesional AD skin (Figure 1a). A similar incremental pattern from healthy to non-lesional and lesional skin, but in opposite direction could be seen for IL1 β and IL18 (Figure 1b-c).

CXCL8 and CCL2, CCL22 and CCL17 were significantly higher in lesional AD skin compared to healthy and non-lesional AD skin (Figure 2a,b,c,e). An opposite pattern was found for CCL4 which was significantly reduced in non-lesional and/or lesional AD skin as compared to healthy skin (Figure 2d). Significant differences between healthy and non-lesional skin, and between non-lesional and lesional skin were found for chemokine CXCL10 (Figure 2f). No significant difference in levels of IL4, IL13, CCL13 and CCL11 was found between healthy skin, lesional and non-lesional AD skin (data not shown).

Although for a few cytokines the level of significance for the comparison between healthy, non-lesional and/or lesional skin differ between skin type II and skin type VI, the overall pattern is similar for these two skin types (Supplementary Figure S1).

Inflammatory immune responses correlate with AD severity

Associations between oSCORAD and immunomodulatory mediators are shown in Table 2. CXCL8 is significantly correlated with oSCORAD in both lesional as well as non-lesional AD skin. CCL17 shows a significant correlation with oSCORAD in non-lesional AD skin and a trend towards a significant correlation in lesional AD skin ($p=0.053$). In lesional AD skin CCL2 is significantly correlated with oSCORAD. The relationship between oSCORAD and biomarkers has further been investigated by multivariate linear regression analysis including a set of biomarkers which were significantly correlated with oSCORAD in non-lesional and/or lesional AD skin (CXCL8, CCL2, and CCL17). Next to these three biomarkers, we included as independent variables FLG mutations and skin types. In non-lesional AD skin, the model with CXCL8, CCL2, CCL17, skin type and FLG mutation shows a significant effect on oSCORAD ($r\ 0.50$, $p<0.05$). In lesional AD skin, only CXCL8 and skin type were significant predictors of oSCORAD ($r\ 0.52$, $p<0.05$). In general, prediction of oSCORAD did not substantially improve as compared to single biomarkers (Table 2).

NMF levels show a trend of gradual decrease from healthy skin to non-lesional and lesional AD skin

NMF levels gradually decreased from healthy skin (median 3.1 (IQR 2.0-4.1)) to non-lesional AD skin (median 2.8 (IQR 1.8-3.6)), with a further decrease in lesional AD skin (median 1.9 mmol per gram protein (IQR Q1-Q3 1.0-3.6)), but no significant differences were found (Figure 3a). Although NMF levels in FLG mutation carriers seemed to be lower than in wild-type subjects, there was no significant difference between the groups (Figure 3b).

NMF levels show different profiles between light and dark skin types

NMF levels were significantly lower in skin type II compared to skin type VI in both lesional and non-lesional AD skin ($p < 0.05$; Figure 4b,c). In healthy skin, NMF levels were not different between skin types (Figure 4a).

When comparing NMF between healthy, non-lesional and lesional skin within skin types, NMF levels within skin type II seem to decrease sequentially in healthy skin to non-lesional and to lesional AD skin (Figure 5a), reaching significance when comparing healthy skin to lesional AD skin ($p < 0.01$). No significant differences could be seen when analyzing only FLG wild-type subjects, although a similar decreasing pattern was seen (Fig 5b). In contrast, in skin type VI, NMF levels were not significantly different between healthy skin and either non-lesional or lesional AD skin (Figure 5c).

AD skin has altered corneocyte texture in both lesional and non-lesional skin

Figure 6 shows representative AFM images of the surfaces of corneocytes sampled from lesional and non-lesional skin of AD children and of healthy skin. DTI values were significantly higher in lesional AD skin compared to both non-lesional AD skin ($p < 0.05$) and healthy skin ($p < 0.01$); Figure 7). DTI values within skin type II showed a sequential increase from healthy skin to non-lesional and lesional AD skin, with a significant difference between healthy skin and lesional AD skin (Figure 8a). There was no significant difference between healthy, non-lesional and lesional AD skin in skin type VI (Figure 8b).

DISCUSSION

The SC is largely responsible for skin barrier function and furthermore harbours a large variety of inflammatory mediators involved in immune responses. Skin biomarkers research in AD children is hampered by invasiveness of sampling techniques such as skin biopsy or blood collection. To our best knowledge, the present study shows for the first time that the minimally invasive method of SC tape stripping is suitable for determination of a large array of skin barrier and immune response biomarkers in AD children, investigating differences between AD skin and healthy control skin. Furthermore, it is the first time that biomarkers differences are investigated between light and dark skin types.

Several immunomodulatory mediators showed significant differences between healthy and atopic skin. IL1 α , an IL-1 cytokine constitutively produced by keratinocytes and present in SC in large amounts showed significant differences between healthy and non-lesional AD skin, and even more pronounced differences between healthy and lesional AD skin. As IL1 α is stored within the corneocyte, skin barrier damage leads to leakage of IL1 α into extracellular space. A similar effect was seen in irritant contact dermatitis which is associated with skin barrier damage (29). IL1 β and IL18, two other IL-1 family cytokines, also showed significant differences between healthy and lesional AD skin and between non-lesional and lesional AD skin. IL18 is associated with *Staphylococcus aureus* colonization, a common feature in AD (30). A decrease of IL1 α and increase of IL1 β and IL18 from healthy skin to non-lesional to lesional AD skin is in agreement with data from a previous study in interstitial fluid in AD which reveals that SC reflects the same cytokine milieu as viable epidermis (31).

Previously, a meta-analysis of literature data found serum CCL17 as the most promising single biomarker of AD severity. Our data showed that CCL17 in non-lesional AD skin was significantly correlated with oSCORAD, and showed a trend towards correlation in lesional AD skin. Next to CCL17, a significant correlation with oSCORAD was found for CXCL8 in both lesional and non-lesional AD skin and for CCL2 in lesional AD skin. Interestingly, levels of CCL17 and CXCL8 showed a correlation with disease severity in non-lesional skin, suggesting that SC samples also provide information on systemic immune response, which is in agreement with a recent study reporting that non-lesional markers generally showed higher and more significant correlations with SCORAD than lesional markers (32).

One of the aims of the present study was to explore possible differences in immune response and skin barrier between different skin types. As the sample size within the skin type groups was small, we performed comparison only between the largest subgroups: light skin type II and dark skin type VI. There was no clear difference in the levels of

immunomodulatory mediator profiles between these two subgroups. However, AD children with skin type II showed lower NMF levels in both non-lesional and lesional AD skin as compared to skin type VI, although both groups had comparable disease severity. As FLG mutations are the main determinant of NMF and they were more prevalent in skin type II, we also performed the comparison in only the children without FLG mutations. When excluding FLG mutation carriers, the NMF levels in skin type II group still showed a trend towards a difference between healthy skin, lesional and non-lesional AD skin, suggesting the effect of AD on the NMF levels in the light skin type. Interestingly, within skin type VI, there was no significant difference in NMF between healthy skin and either non-lesional or lesional AD skin. Consistent with our study where only one carrier of FLG mutations within skin type VI has been detected, different studies have reported that the prevalence of FLG mutations in African AD patients is very low compared to European Caucasians (33,34). Although rare population-specific mutations cannot be excluded, it is not likely that they would have largely influenced the results on NMF levels found in the present study. Another skin barrier parameter, DTI showed a sequential increase from healthy skin to non-lesional and lesional AD skin within skin type II with a significant difference between healthy skin and lesional AD skin. However, this pattern of gradual increase was not present in skin type VI and no significant differences were seen. In general, data on differences in skin barrier between different skin types and ethnicities is scarce. Several studies reported differences in TEWL, lipid content, ceramides, intercellular cohesion and skin pH in African American children as compared to Caucasians and East Asians. As TEWL, intercellular cohesion and skin pH are known to be affected by NMF, (5, 7, 35) it would be interesting to investigate whether higher NMF in children of skin type VI found in the present study are correlated to these important parameters of the skin barrier function.

Our study has several limitations; the sample size was relatively small and not powered on differences between skin types, accordingly, subgroup analyses in all skin types were not possible. Since there was a significant difference in sampling period, as the majority (76%) of healthy controls were seen during summer compared to 49% of AD children. UV exposure may have influenced NMF levels and DTI values in controls (36).

In conclusion, collecting SC samples with minimal invasive tape strip method is suitable for determination of a large array of immunomodulatory mediators and skin barrier biomarkers in AD children. CXCL8, CCL2, and CCL17 are the most promising biomarkers to assess the severity of disease. This study shows that immunomodulatory mediator profiles are similar in light and dark skin types, but that NMF levels and DTI values differ between AD children with skin type II and skin type VI. This may suggest a different role for skin barrier dysfunction in the pathogenesis of AD between light and dark skin types.

Ethical statement

The study was conducted according to principles of the Declaration of Helsinki and was approved by the Ethics Committee of the Academic Medical Centre (AMC) and approved by the Medical Ethics Review Committee of the AMC Amsterdam (METC 2013_372). Written informed consent was obtained from both parents/legal guardians of all children.

Conflict of interest

B.L., as indicated by the affiliations, is leading the strategic alliance between the University Medical Centre Utrecht/Wilhelmina Children's Hospital and Nutricia Research and is employed by Nutricia Research. All other co-authors declare no potential conflict of interest.

Author contributions

Performed the study; L.H., D.H., B.D., A.S., M.M. Designed and supervised the study; S.K., A.S., M.M., W.A. Participated in data collection, analyses and Interpretation; L.H.; D.H.; Q.H.; B.D.; I.J.; B.L.; S.K.; A.S.; M.M.; W.A.; C.R.; W.L.; L.C. Drafted the manuscript L.H.; D.H.; Q.H.; B.D.; S.K.; A.S.; M.M.

All authors revised and approved the final version of the manuscript.

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Figure legends

Figure 1 Levels of IL-1 cytokines (IL-1a, IL-1b and IL-18) in healthy skin of control children (CTRL green/●, n=49) and in AD children with non-lesional and lesional skin (NL blue/■, n=53 and L red/▲, n=24). The differences in IL-1 cytokines between healthy and AD skin were tested by Mann Whitney U test. Wilcoxon Signed Rank test was used to test differences between the unaffected and affected AD skin. Statistically significant differences; Benjamini-Hochberg corrected p-values are indicated with asterisks: * p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 2 Cytokine and chemokines levels in healthy skin of control children (CTRL green/●, n=49) and AD children with non-lesional or lesional skin (NL blue/■, n=53 and L red/▲, n=24). The differences in chemokine levels between healthy and AD skin were tested by Mann Whitney U test. Wilcoxon Signed Rank test was used to test differences between non-lesional and lesional AD skin. Statistically significant differences; Benjamini-Hochberg corrected p-values are indicated with asterisks: * p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure S1 immunomodulatory mediators within skin type II and VI in healthy skin of control children (CTRL green/●), non-lesional AD skin (NL blue/■) and lesional AD skin (L red/▲) are shown. The differences in chemokine levels between healthy and AD skin were tested by Mann Whitney U test. Wilcoxon Signed Rank test was used to test differences between non-lesional and lesional AD skin. Statistically significant differences; Benjamini-Hochberg corrected p-values are indicated with asterisks: *p<0.05; **p<0.01.

Figure 3 Differences in the natural moisturizing factor levels (NMF) in stratum corneum (SC) in healthy skin of control children and in non-lesional and lesional skin of AD children. The results of n=49 healthy skin of control children (CTRL ●), n=53 non-lesional AD skin (NL ■) and n=25 lesional AD skin (L ▲) are shown. The red marked symbols represent children with FLG-loss-of-function mutation(s); CTRL n=3, NL n=14, L n=6. Data are shown as median with interquartile ranges (IQR Q1-Q3). A Mann Whitney U test (MWU-test) was used to investigate significant difference between healthy skin versus unaffected AD skin, as the Wilcoxon matched-pairs signed rank test (W-test) was used to investigate differences between the paired samples of unaffected versus affected AD skin. Statistically significant differences; Benjamini-Hochberg corrected p-values are indicated with asterisks: *p<0.05.

Figure 4 Natural moisturizing factor levels (NMF) in stratum corneum (SC) of skin type II and VI in a) healthy skin of control children (CTRL● n=29), b) non-lesional AD skin (NL■ n=32) and c) lesional AD skin (L▲ n=14). The red symbols represent the children with FLG loss-of-function mutation(s). Data are shown as median ± interquartile ranges. Statistically significant differences; Benjamini-Hochberg corrected p-values, between skin types within the groups are indicated with asterisks: **p<0.01.

Figure 5 Natural moisturizing factor levels (NMF) in the stratum corneum (SC) within skin type II and VI in healthy skin of control children (CTRL ●), non-lesional AD skin (NL ■) and lesional AD skin (L ▲) are shown. n=45 in skin type II, n=29 in skin type II without FLG loss-of-function mutation(s) and n=30 in skin type VI. The red symbols represent the children with FLG loss-of-function mutation(s). Data are shown as median ± interquartile ranges. A Mann Whitney U test (MWU-test) was used to investigate significant difference between healthy skin versus unaffected AD skin, as the Wilcoxon matched-pairs signed rank test (W-test) was used to investigate differences between the paired samples of unaffected versus affected AD skin. Statistically significant differences; Benjamini-Hochberg corrected p-values are between skin types within the groups are indicated with asterisks: *p<0.05; **p<0.01.

Figure 6 Representative AFM images (20 μm) of the surfaces of corneocytes sampled from lesional (a) and non-lesional (b) skin of children with AD and c) healthy skin of control children. On visual inspection, the number of nano-size protrusions was the highest in AD lesional skin (a).

Figure 7 The Dermal Texture Index (DTI) in the stratum corneum (SC) in healthy skin of control children (CTRL●, n=16), in non-lesional AD skin (NL■, n=21) and in lesional AD skin (L▲, n=13). The red symbol represents the children with FLG mutation(s). Data are given as median ± interquartile ranges. The difference between healthy subgroup and AD subgroups was tested by Mann Whitney U test. Wilcoxon Signed Rank test was used to investigate differences between the paired samples of non-lesional versus lesional AD skin. Statistically significant difference; Benjamini-Hochberg corrected p-values are indicated with asterisks: **p<0.01, **** p<0.0001).

Figure 8 The Dermal Texture Index (DTI) in the stratum corneum (SC) within skin type II and VI in healthy skin of control children (CTRL ●), non-lesional AD skin (NL ■) and lesional AD skin (L ▲) are shown. a) skin type II (n=23); b) skin type VI (n=23). The red symbols represent the children with FLG loss-of-function mutation(s). Data are given as median ± interquartile ranges. The difference between healthy subgroup and AD subgroups was tested by Mann Whitney U test. Wilcoxon Signed Rank test was used to investigate differences between the paired samples of non-lesional versus lesional AD skin. Statistically significant difference; Benjamini-Hochberg corrected p-values are indicated with asterisks: *p< 0.05, **p<0.01).

Table 1 Patient characteristics

Characteristic	Healthy skin controls (<i>n</i> = 50)	AD Children (<i>n</i> = 53)	P-values
Male, <i>n</i> (%)	30 (60.0)	25 (47.2)	
Age (months)			
mean (\pm SD)	75.9 (41.9)	41.9 (40)	0.02
range	13-179	5-140	
Skin type, <i>n</i> (%)			
I	3 (6.4)	3 (5.7)	
II	20 (36.2)	17 (32.1)	
III	3 (6.4)	5 (9.4)	
IV	13 (27.7)	10 (18.9)	
V	2 (4.3)	3 (5.7)	
VI	9 (19.1)	15 (28.3)	
Objective SCORAD score (mean \pm SD)	0 (0)	17.6 (9.7)	<0.0001
FLG carriers, <i>n</i> (%)	3 (6)	14 (27)	0.005
In skin type I	0	1 (2)	
In skin type II	3 (6)	9 (17)	
In skintype III	0	2 (4)	
In skintype IV	0	1 (2)	
In skintype V	0	0	
In skintype VI	0	1 (2)	
Season of tape stripping visit (<i>n</i> , %)			0.004
Spring	5 (10)	17 (32)	
Summer	38 (76)	26 (49)	
Autumn	4 (8)	1 (2)	
Winter	3 (6)	9 (17)	

AD: atopic dermatitis, Age and objective SCORAD score are expressed as mean with standard deviation, skin type and male ratio are expressed as frequency: *n* is the number of children in each group; with proportion in %, A Mann Whitney U test or the chi-square test for was used to investigate significant difference between the two groups: $p < 0.05$.

Table 2 Overall association of immunomodulatory mediators with AD severity

Immunomodulatory mediator	Non-lesional AD skin n=53		Lesional AD skin n=24	
	Spearman r	BH corrected p- value	Spearman r	BH corrected p- value
IL1 cytokines				
IL1 α	-0.194	0.299	-0.455	0.089
IL1 β	0.082	0.715	-0.365	0.181
IL18	0.121	0.569	0.437	0.104
Inflammatory mediators				
CXCL8	0.391	0.024	0.533	0.041
Th2 cytokines				
IL13	0.124	0.558	0.047	0.881
IL4	-0.005	0.998	-0.388	0.145
Chemokines				
CCL2	0.291	0.104	0.522	0.044
CCL13	0.176	0.352	-0.322	0.250
CCL22	0.272	0.133	0.311	0.267
CCL17	0.417	0.014	0.504	0.053
CCL4	0.241	0.183	0.149	0.658
CXCL10	0.141	0.490	0.398	0.142
CCL11	0.063	0.776	-0.434	0.104

Correlation is considered significant if Benjamini-Hochberg corrected p-values < 0.05 (2-tailed)







